EGb 761 Protects Cardiac Microvascular Endothelial Cells against Hypoxia/Reoxygenation Injury and Exerts Inhibitory Effect on the ATM Pathway

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Ginkgo biloba extract (EGb 761) has been widely used clinically to reduce myocardial ischemia reperfusion injury (MIRI). Microvascular endothelial cells (MVECs) may be a proper cellular model in vitro for the effect and mechanism study against MIRI. However, the protective effect of EGb 761 on MVECs resisting hypoxia/reoxygenation (H/R) injury is little reported. In this study, H/R-injured MVECs were treated with EGb 761, and then the cell viability, apoptosis, ROS production, SOD activity, caspase-3 activity, and protein level of ATM, γ-H2AX, p53, and Bax were measured. ATM siRNA was transfected to study the changes of protein in the ATM pathway. EGb 761 presented protective effect on H/R-injured MVECs, with decreasing cell death, apoptosis, and ROS, and elevated SOD activity. Next, EGb 761 could inhibit H/R-induced ATM, γ-H2AX, p53, and Bax in a dose-dependent manner. Moreover, ATM siRNA also could inhibit H/R-induced ATM, γ-H2AX, p53, and Bax. Overall, these findings verify that EGb 761 protects cardiac MVECs from H/R injury, and for the first time, illustrate the influence on the ATM pathway and apoptosis by EGb 761 via dampening ROS.

Keywords: EGb 761, myocardial ischemia reperfusion injury, microvascular endothelial cells, hypoxia/reoxygenation (H/R) injury, ataxia-telangiectasia mutated (ATM)

Introduction

Myocardial ischemia reperfusion injury (MIRI) refers to the worsened metabolic disorder and ultrastructural changes of ischemic myocardium by restoring blood flow, including microvascular obstruction (MVO), endothelial cell sloughing within the intramyocardial capillaries, and myocyte necrosis [1, 2]. Thus, microvascular maintenance and integrity during MIRI may contribute significantly to myocyte survival and myocardial function. Owing to the direct exposure to MVO, endothelial dysfunction initiates the pathological process and plays an integral role, so that it is important to protect the endothelium against MIRI. On account of the heterogeneity and organ specificity of endothelial cells [3], microvascular endothelial cells (MVECs) may be more proper to mimic the actual pathological state and be used in drug research for in vitro cellular assays. Ginkgo biloba extract (GBE) is a tolerable and safe herbal supplement that exerts a significant increase in peripheral and cerebral blood flow and microcirculation [4–6]. GBE has shown highly effective cytoprotection in clinical treatment of cardiovascular diseases, including MIRI [6]. EGb 761 is a standardized GBE used widely in clinic, the constituents of which include approximately 24% flavone glycosides, 6% terpene lactones, and other chemicals [7]. These major bioactive components exert various pharmacological activities against MIRI, including free radical scavenging, inhibition of platelet activating factor, and protection of MVECs. Flavone has been verified for its cardioprotective actions in various MIRI models [8, 9]. Terpene lactone also exhibits anti-excitotoxicity, radical scavenging capacity, and regulation of mitochondrial gene expression [10].
ATM (ataxia-telangiectasia mutated) kinase can be activated by reactive oxygen species (ROS)-induced DNA damage, to trigger the downstream signals, such as cell apoptosis and DNA repair. This process can stabilize the genome and prevent wrong genetic information in the daughter cells. Extensive studies have reported that mutant ATM overexpresses in lymphoid malignancies and solid tumors [11], whereas the pathological effect of ATM in endothelial cells is not clear. Herein, we speculated whether ATM could express increasingly in hypoxia/reoxygenation (H/R)-injured MVECs, and whether EGb 761 could take effect in this process.

Although numerous studies have proved the pharmacological effect and mechanism of EGb 761 on MIRI both in vitro and in vivo, very little laboratory evidence has been provided on the effect of EGb 761 on MVECs. In this present study, the protective effect and underlying mechanism of EGb 761 were investigated on H/R-injured MVECs, and whether EGb 761 could take effect in this process.

Materials and Methods

MVECs Isolation, Culture, and Exposure to H/R Injury

MVECs were isolated from rat hearts with modified trypsin treatment [12]. In brief, the left ventricular anterior walls were isolated from the rat hearts, flushed and rinsed with PBS, then stripped the surface layers of endocardium and epicardium. The remaining myocardial tissues were cut into small pieces, and incubated with 0.25% trypsin-EDTA and 0.2% collagenase type II for 15 min at 37°C. The detached endothelial cells were harvested by flowing through a filter and centrifugation, and then cultured to reach 90% confluence in rat endothelial cell growth medium (REGM-2; Sigma, USA) in a normoxic atmosphere of 21% O₂.

During the hypoxic period, MVECs were placed in humidified airtight incubation chambers and perfused with air (0% O₂, 5% CO₂ and balanced N₂), and then maintained at 37°C in a tissue culture incubator for 2 h. During the reoxygenation period, the cells were renewed with normoxic culture with 21% O₂.

Immunofluorescence Staining

Factor VIII was used to identify the endothelial cells. When MVECs grew into a confluent monolayer, the cells were fixed for 10 min with 4% paraformaldehyde at room temperature, washed three times with PBS, and then incubated in 5% BSA with 0.5% Triton X-100 for 30 min. After washing with PBS, the cells were incubated with primary antibody (1:100; Santa Cruz, USA) overnight at 4°C, and on the next day followed by a FITC-labeled secondary antibody (Boster, China) staining for 2 h in dark. After enough washing, positive cells were observed and counted under fluorescence microscopy. MVECs with more than 90% positive were used for subsequent assays.

ATM siRNA Transfection in H/R-Injured MVECs

To reduce the cytotoxicity by transfection reagent, Lipofectamine 3000 reagent (Invitrogen, USA) was used to knock down ATM following the manufacturer’s instruction. The siRNA nucleotide sequence was GGC UAU UCA GUA UGC CAG A. After 24 h transfection, MVECs were treated with H/R and EGb 761.

Cell Growth Using the MTT Assay

MVECs were trypsinized, counted, and seeded in a 96-well plate (5,000 cells/well/50 μl). Only 50 μl of medium per well was added to reduce the diffusion distance during the hypoxic period. After 2 h severe hypoxia with 0% O₂, the cells were reoxygenated with 21% O₂ for 24 h. During the hypoxia and reoxygenation period, 100 mg/ml of EGb 761 was used to treat MVECs. Following H/R injury in vitro, 10 μl of MTT was added to the medium for 4 h incubation, and then the medium was discarded and replaced with 100 μl of DMSO. The absorbance was read at 490 nm.

Cell Apoptosis Analysis by Flow Cytometry

Briefly, after reoxygenation, cells were harvested, washed, and incubated with a solution of Annexin V-FITC for 20 min and then PI (50 μg/ml) for 10 min. All staining operations were carried out on ice and in the dark. Then, the cells were analyzed with a flow cytometer (Becton Dickinson, USA).

Measurement of ROS

ROS production by MVECs was determined by 2',7'-dichlorodihydrofluorescein diacetate assay (H2DCFDA; Invitrogen, USA). MVECs were washed, trypsinized, pelleted, and resuspended with PBS at 1 × 10⁶ cells/ml, and incubated with 10 μM H2DCFDA for 20 min at 37°C in the dark. Then, fluorescence at 520 nm was detected to represent ROS production.

Determination of Superoxide Dismutase (SOD) Activity

The activity of SOD was measured for the inhibition of superoxide anions. Cells were lysed by 1% TritonX-100 for 30 min on ice and then centrifuged at 12,000 x g, 4°C for 10 min. Protein concentrations of the supernatants were determined by the BCA protein assay (Beyotime, China). Aliquots of 30 μl supernatants were incubated with reactive solutions at 37°C in a water bath for 40 min. The absorbance was spectrophotometrically assessed at 550 nm. SOD activities (U/mg protein) were calculated using the equation provided by the manufacture (Jiancheng Bioengineering Institute, China).

Western Blot Assay

Cell lysates were prepared in cell lysis buffer (Sigma, USA) containing 1 mM PMSF. The total protein content was measured...
by BCA protein assay. The equal amounts of protein were electrophoresed on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked, incubated for 2 h with primary antibodies, and subsequently incubated with secondary antibody (Boster, China) for 2 h. The blots were visualized using ECL detection reagents (Beyotime, China). The following antibodies were used: ATM, p53, Bax, and GAPDH were purchased from Santa Cruz (USA), and γ-H2AX was purchased from Cell Signaling Technologies (USA).

**Caspase-3 Activity Assay**

After ATM siRNA transfection, following with 2 h hypoxia and 24 h normoxia, the cells were trypsinized to be collected and lysed in lysis buffer for 15 min on ice (2 × 10⁶ cells :100 μl lysis buffer), and then centrifuged to get the supernatant. The supernatant samples were mixed with the substrate of Ac-DEVD-pNA. After 2 h incubation in 37°C, the optical density was detected.

**Statistical Analysis**

Statistical software SPSS 15.0 was used for statistical analysis. Data was expressed as the mean ± SD. Differences between independent groups were analyzed using the Student’s t-test.

**Results**

**Identification of MVECs**

Although in early passages some non-endothelial cells were isolated and collected, the medium with enriched heparin and growth factors could mainly support endothelial cell proliferation and inhibit the growth of contaminant cells. Under the microscope, MVECs were factor VIII-positive with a percentage over 90% (Fig. 1A).

**EGb 761 Facilitated MVECs Survival and Suppressed Apoptosis under H/R Injury**

To observe the protective effect of EGb 761, we compared the cell proliferation and apoptosis of H/R-induced MVECs with or without EGb 761 treatment. Both at 2 h of hypoxia period and 24 h of reoxygenation period, the absorbance values were detected by MTT assay to display the cell viability. As shown in Fig. 1B, severe hypoxia to 0% O₂ exhibited the anti-proliferation effect to MVECs and decreased the proliferation ability even though restoring oxygen again, which verified the injury by H/R. However, EGb 761 obviously protected cell survival from H/R injury without significant difference from normoxic MVECs at both 2 h of hypoxia and 24 h of reoxygenation. Then, we confirmed the protective effect of EGb 761 on H/R-provoked apoptosis by flow cytometry assay. Dot plots in the upper right quadrant (Annexin V⁺/PI⁺) represented late apoptosis states of the cells. As shown in Fig. 1C, H/R led to a significant increase in the proportion of apoptotic cells to 25.5 ± 6.7%, whereas EGb 761 treatment markedly inhibited the apoptosis of MVECs to 12.2 ± 2.6%.

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*Fig. 1. Protective effect of EGb 761 on hypoxia/reoxygenation (H/R)-induced microvascular endothelial cells (MVECs). (A) Immunofluorescence shows the Factor VIII expression in cultured MVECs from rat hearts. The left photo shows the MVECs morphology and the right photo presents the FITC-factor VIII staining of MVECs. (B) MTT assay presents the survival protection by EGb 761 under H/R injury. (C) EGb 761 partially reverses H/R-induced apoptosis of MVECs. *: p < 0.05 vs. H/R MVECs; #: p < 0.05 vs. normoxic MVECs.*
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EGb 761 Notably Attenuated the Oxidative Damage and Enhanced SOD Activity in MVECs Induced by H/R Injury

It has been reported that excessive generation of ROS plays a major role in the initiation of apoptosis during acute myocardial infarction. The antioxidative effect of EGb 761 was verified in this study. As presented in Table 1, the intracellular ROS levels in MVECs in response to H/R injury increased about 2-fold compared with normoxic MVECs, which could be significantly dampened by treatment with EGb 761. Thus, this result suggests EGb 761’s powerful ability in ROS scavenging.

As presented in Table 1, SOD (an eliminator of free radicals) activity of MVECs decreased under H/R condition, which

Table 1. The antioxidative effect of EGb 761 on H/R-injured MVECs.

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<th>Normoxic MVECs</th>
<th>H/R MVECs</th>
<th>H/R MVECs+EGb 761</th>
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<tr>
<td>ROS production (%)</td>
<td>100.0 ± 12.44</td>
<td>178.34 ± 15.56$^b$</td>
<td>119.24 ± 13.21$^a$</td>
</tr>
<tr>
<td>SOD activity (U/mg protein)</td>
<td>39.43 ± 1.48</td>
<td>23.25 ± 1.46$^b$</td>
<td>32.33 ± 1.62$^a$</td>
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$^a$p < 0.05 vs. H/R MVECs; $^b$p < 0.05 vs. normoxic MVECs.

Fig. 2. Western blot assay showed the ATM, γ-H2AX, p53, and Bax regulation in H/R-injured MVECs by EGb 761. (A) Representative immunoblots; (B) densitometric analysis. n = 5, mean ± SD. *: p < 0.05, vs. H/R group.
presented a decreased ability for scavenging free radicals. However, EGB 761 could improve the situation to enhance SOD activity.

**EGB 761 Reduced H/R-Induced p-ATM and Its Downstream Molecules in MVECs**

The ATM pathway has an important function controlling the cellular response to DNA damage induced by exogenous stimuli. We therefore hypothesized and compared the phosphorylation status of ATM in H/R-injured MVECs treated with serial dosage of EGB 761, or the antioxidants, N-acetyl-L-cysteine (NAC).

Western blot analysis (Fig. 2A) indicated that compared with normoxic MVECs, H/R injury increased the protein expression of phosphorylated ATM (p-ATM), γ-H2AX, p53 (p-ATM downstream molecule), and Bax (a pro-apoptotic protein). It indicated that the ATM pathway participated in the signal transduction and induced apoptosis in the pathogenesis of H/R-injured MVECs. NAC, a ROS scavenger, decreased the levels of p-ATM, γ-H2AX, and p53, and consequently downregulated Bax (Fig. 2A). The results together supported that ROS by H/R injury mediated the activation of the ATM pathway and cell apoptosis.

However, EGB 761 could reverse this status to inhibit the expression of four proteins in a dose-dependent manner (Fig. 2B), which revealed EGB 761 might protect MVECs from H/R injury throughout the ATM pathway.

**ATM siRNA Inhibited the Apoptosis in H/R-Induced MVECs**

As shown in Figs. 3A and 3B, pre-treatment with ATM siRNA could significantly inhibit the p-ATM protein level, and reduce the expression level of γ-H2AX and p53. We further found that ATM knockdown also decreased Bax protein, suggesting that ATM regulated apoptosis in H/R-induced MVECs. Next, we investigated the changes in caspase-3 activity. Fig. 3C indicated that both EGB 761 and ATM knockdown could notably inhibit caspase-3 activity. These data suggested that the ATM pathway participated in regulation of apoptosis in H/R-induced MVECs.

Interestingly, it seemed that the reduced levels of p53, Bax, and caspase-3 activity by ATM knockdown were less than those by EGB 761, indicating that there might be other pathways triggered by EGB 761.

**Discussion**

The microvascular endothelium represents the inner cellular lining separating the microcirculation from underlying tissues. These endothelial cells, consistent with those from the macrovascular system, play a significant role in many physiological functions such as transport, permeability, synthetic and secretory processes, and homeostatic balance. However, the MVECs seem to be more sensitive to pathogenic factors and more precise to modulate cellular functions. McDouall et al. [13] reported that heart MVECs showed 100-fold higher sensitivity of MHC class II production than HUVECs or endothelial cells from large vessels. Additionally, endothelial phenotypes differ between species, organs, and vascular beds. Endothelial cells from different organs demonstrate unique structural and functional properties as well as distinct developmental

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Fig. 3. Inhibitive effect of ATM siRNA on H/R-injured MVECs against ATM pathway activation and apoptosis. (A) Representative immunoblots; (B) densitometric analysis. (C) Caspase3 activity in H/R-injured MVECs treated with EGB 761 or ATM siRNA. n = 5, mean ± SD. *: p < 0.05, vs. H/R group.
programs, roles in pathophysiology, and potential for targeted therapy in patients with vascular diseases [14, 15]. MVECs may therefore be a valuable cell source and have been used for drug discovery and pathogenesis research. In this study, over 90% Factor VIII-positive MVECs were achieved, which presented the endothelial source and the successful isolation and culture. It was the base for the following studies.

During MIRI, oxidative damage emerges as a main cause of endothelial dysfunction in the heart [16]. Notable ROS increase to a toxic level can initiate lipid peroxidation, oxidize protein to inactive states, and cause DNA damage, as well as stimulate various signal pathways to induce several biological effects, such as pro-apoptosis, pro-inflammation, and anti-proliferation [17]. The studies in vivo or in clinic have presented similar results. EGb 761 administration can attenuate MIRI and improve the myocardial ultrastructure of ischemia reperfusion in rats [18]. In an epidemiology study of diets rich in flavonoids, such as EGb 761, could affect endothelium dysfunction to reduce the risk of cardiovascular disease [19]. Our studies reveal that EGb 761 can directly inhibit H/R-induced ROS and cell apoptosis, and also elevate SOD activity in MVECs. These results not only exhibit the biological responses of MVECs in H/R-injured pathology, but demonstrate the protective effect of EGb 761 in an in vitro model of endothelial dysfunction against MVECs.

Chemical agents and physical events, including H/R, may injure cells, determining an increase in oxidative stress and genotoxic damage to activate the ATM/p53 cascade and biological effects, such as cell cycle arrest and cellular senescence, which are essential for DNA damage repair or programmed cell death. It has been found that ROS-induced DNA damage can activate ATM kinase by increased autophosphorylation [20], in turn to trigger phosphorylation of \( \gamma \)-H2AX [21], following the initiation of phosphorylated p53 substrate protein [22]. Phosphorylated p53 regulates Bax expression by binding the Bax gene promoter [23] to mediate apoptosis. Although extensive studies about ATM focus on the field of lymphoid malignancies and solid tumors [11], a few studies have reported the participation of the ATM pathway on endothelial cells. Chiang et al. [24] reported that chemical-provoked ROS stimulates ATM/p53-modulated Fas and DR4/DR5 signals to promote apoptotic death in HUVECs [24]. Using cDNA microarray, Shen [25] discovered that gene expression of ATM and TNFSF10 was boosted by hypoxia in HUVECs, whereas obvious downregulation of these two genes was achieved after treatment with EGb 761. Additionally, it has been reported that phosphorylated p53 expression increases in H/R-treated MVECs [26] from other animal organs [27], or patients undergoing MIRI [28]. Bax also can be upregulated in heart exposed to intermittent hypoxia [29]. In this present study, given the observation of elevated ROS generation (Table 1) and cell apoptosis (Fig. 1C) in H/R-injured MVECs, we therefore examined whether ATM and p53 were involved in the process. For the first time, it is revealed that ATM and ATM-related cascade molecules are expressed increasingly in heart MVECs induced by H/R injury. Next, NAC decreases the expression of ATM, \( \gamma \)-H2AX, p53, and Bax (Fig. 2), suggesting ROS-mediated activation of the ATM pathway. Moreover, ATM knockdown notably inhibits caspase-3 activity and Bax expression (Fig. 3), which suggests that the ATM pathway participates in regulation of apoptosis in H/R-induced MVECs, whereas treatment with EGb 761 reverses the protein upregulation and caspase-3 activation in a dose-dependent manner.

The data in this study indicates that the DNA damage by ROS possibly provokes the phosphorylation of ATM and in turn induces cell apoptosis via p53-transducted caspase-3 activation. However, EGb 761 can inhibit ROS production to attenuate the DNA damage accompanying with alleviating phosphorylated ATM and ATM-related downstream molecules, which in turn presents the decreased cell apoptosis.

In conclusion, EGb 761 can protect MVECs viability, including (i) decreasing ROS production, (ii) increasing SOD activity, (iii) attenuating cell apoptosis, and (iv) influencing the ATM pathway with downregulated ATM, \( \gamma \)-H2AX, p53, and Bax.

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